Redox Status and Protein Binding of Plasma Homocysteine and Other Aminothiols in Patients With Early-Onset Peripheral Vascular Disease

Homocysteine and Peripheral Vascular Disease

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Abstract Elevated total homocysteine (Hcy) in plasma is an independent risk factor for early-onset vascular disease in the coronary, cerebral, and peripheral arteries. Different forms of Hcy, and their relation to other aminothiols in plasma, have not been investigated in patients with vascular disease. We therefore investigated 65 patients (35 men and 30 women) operated on for peripheral arterial disease at <50 years of age and 65 age- and sex-matched control subjects. Total, reduced, oxidized, and protein-bound Hcy, cysteine (Cys), and cysteinylglycine (CysGly) were measured 0 to 11 years (mean, 6 years) postoperatively, in the fasting state, and after a standard methionine loading dose that caused a transient increase in reduced, oxidized, and protein-bound Hcy. All forms of Hcy and Cys, except reduced Cys, were higher in fasting patients than fasting control subjects. A similar difference between the groups was observed after methionine loading. The levels of most Hcy forms both during fasting and after methionine loading were related to smoking, but multivariate analysis showed that the difference between patients and control subjects could not be explained by smoking alone. Notably, reduced Cys and the reduced/total ratio for Cys were significantly higher in control subjects than in patients, both during fasting and after methionine loading. In both groups, the redox status and protein binding of the various aminothiols in plasma were interactive, as demonstrated by positive correlations between their reduced/total ratios and by a decrease in protein-bound Cys when protein-bound Hcy was elevated during methionine loading. Serum folate and to a lesser degree serum cobalamin and vitamin B_6 were predictors of oxidized and protein-bound Hcy in some patients and control subject subgroups. Thus, reduced, oxidized, and protein-bound Hcy are elevated and reduced Cys is decreased in patients with peripheral arterial disease. Reduced Hcy acts as a pro-oxidant in vitro and is a possible atherogenic agent, whereas reduced Cys may be a protective agent as a part of the antioxidant defense system. The protein binding and redox status of different plasma aminothiols are interactive in a way suggesting ongoing redox cycling and disulfide exchange reactions. Thus, Hcy is one component in a complex system. (***Arterioscler Thromb Vasc Biol.* 1995;15:232-240.**)

Key Words • risk factor • cysteine • cysteinylglycine • methionine loading • vitamins

There is clinical and epidemiological evidence suggesting that elevated levels of homocysteine (Hcy) in blood are associated with vascular disease. Patients with various inborn errors of Hcy metabolism, causing extremely high levels of homocyst(e)ine in blood and urine, ie, homocystinuria, develop premature cardiovascular disease in early adolescence and even in childhood.[1] About 2500 cardiovascular patients and a comparable number of control subjects have been investigated in approximately 20 retrospective and two prospective[2,3] epidemiological studies. These investigations show that a moderately elevated concentration of Hcy in plasma, so-called hyperhomocysteinemia, is an independent risk factor for premature vascular disease localized to the coronary, cerebral, and peripheral vessels.[4-6]

Hcy is a sulfur amino acid and a product of transmethylation. It is either degraded to cysteine (Cys) or remethylated to methionine. The former reaction is catalyzed by the vitamin B_6–dependent enzyme cystathionine β-synthase (EC 4.2.1.22), whereas in most tissues, remethylation is catalyzed by methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase, EC 2.1.1.13), which uses methyltetrahydrofolate as the methyl donor and cobalamin as cofactor.[7] Thus, Hcy metabolism is linked to the metabolism and function of folates, cobalamin, and vitamin B_6, and this explains why deficiencies of these vitamins are common causes of hyperhomocysteinemia.[8]

In plasma from healthy subjects, most Hcy (70% to 80%) is protein bound, and the remaining is acid-soluble free Hcy.[9] Most free Hcy exists as Cys-Hcy mixed disulfide.[10] The sum of all Hcy forms in plasma has been termed total Hcy, which is a robust parameter not affected in vitro by disulfide exchange reactions and redistribution between Hcy forms.[8,11] Clinical studies on plasma Hcy in patients with cardiovascular disease,[5,6] vitamin deficiencies,[12] or other disease states[8] are usually based on measurements of total Hcy.

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TABLE 1. Characteristics of 65 Patients With Peripheral Vascular Disease and 65 Control Subjects

	Patients		Control Subjects	
	Men (n=35)	Women (n=30)	Men (n=34)	Women (n=31)
Age, y*	47 (36-59)	50 (40-62)	47 (35-60)	50 (38-61)
Serum lipids, mmol/L†				
Total cholesterol	6.3 (5.8-6.8)	6.6 (6.1-7.2)	6.1 (5.7-6.5)	5.8 (5.3-6.3)
HDL cholesterol	1.1 (0.9-1.3)	1.2 (1.1-1.3)	1.3 (1.1-1.4)	1.5 (1.3-1.6)
LDL cholesterol	4.0 (3.5-4.5)	4.6 (4.2-5.1)	4.1 (3.8-4.5)	3.7 (3.2-4.2)
Triglycerides	2.1 (1.7-2.6)	1.7 (1.5-2.0)	1.3 (1.1-1.7)	1.1 (0.9-1.3)
Smoking, n (%)				
Current	17 (49)	18 (60)	9 (27)	10 (32)
Previous	35 (100)	28 (93)	22 (65)	15 (48)
Diabetes, n (%)	4 (11)	3 (10)	0 (0)	1 (3)
Hypertension, n (%)	8 (23)	7 (23)	2 (6)	6 (19)

*Median and range.
†Mean and 95% confidence interval of the mean in parentheses.

Since Hcy in blood is rapidly oxidized and is associated with plasma proteins, assessment of its redox status and protein binding in human plasma requires immediate derivatization of the reduced Hcy and separation of the free and bound forms. We have recently developed such a method, which measures reduced, oxidized, and protein-bound Hcy, Cys, and cysteinylglycine (CysGly) in human plasma.[13] We have determined these parameters in healthy subjects given a peroral methionine[14] or Hcy[15] load and in plasma from patients with homocystinuria[16] and cobalamin deficiency,[17] ie, states characterized by marked elevation of plasma Hcy. These data suggest that the concentration, protein binding, and redox status of Hcy induce secondary effects on redox status of other aminothiols in plasma.

The purpose of the present study was first to uncover possible aberrations in the concentration, redox status, and protein binding of plasma Hcy but also in related aminothiols in a population with early-onset vascular disease. A second objective was to investigate whether the dynamic relations that exist between aminothiol forms under conditions of marked elevation (>30 μmol/L) of plasma Hcy[14-17] also exist in healthy persons with normal plasma Hcy levels and in vascular disease patients consistently reported to have moderately elevated plasma Hcy compared with control subjects.[5,6] Assessment of redox status and protein binding of several aminothiols in plasma may create a more differentiated picture of these components as possible risk factors for premature vascular disease. In addition, knowledge of remote effects of hyperhomocysteinemia on the levels and redox status of other aminothiols may guide future research on the mechanisms behind the vascular lesions.

Methods

Patients and Control Subjects

In the period 1979 to 1990, 82 patients had vascular reconstruction for peripheral vascular disease (excluding trauma and emboli) before the age of 50 years at the Department of Surgery of Karolinska Hospital and St Görans Hospital, Stockholm, Sweden. This represents about 3% of the total population admitted to these departments for operation. Of the 82 patients, 9 died (all from cardiovascular disease), 3 emigrated, 3 refused to participate in the study, and 1 could not be traced. The remaining 66 patients were available for follow-up.

We investigated 65 patients, 35 men and 30 women. The median age at the onset of symptoms was 40 years (range, 20 to 49 years); at surgery, 44 years (range, 21 to 49 years); and at follow-up, 49 years (range, 36 to 62 years). They had infrainguinal lesions (17 patients), suprainguinal lesions (28 patients), or multilevel disease (12 patients). Four patients were operated on for abdominal aortic aneurysms, 2 patients for renal artery stenosis, and 2 patients for carotid artery stenosis. They were compared with 65 randomly selected age- and sex-matched control subjects selected from the population register. Characteristics of patients and control subjects are summarized in Table 1.

The participants provided their written informed consent, and the protocol was approved by the ethics committee at the Karolinska Hospital.

Methionine Loading and Blood Sampling

The subjects recruited to the study were called in and investigated in groups of 6 to 8 subjects, and the number was usually equally divided between patients and control subjects. They were subjected to methionine loading by oral administration of methionine (100 mg/kg body wt) in 200 mL of orange juice. Blood samples were collected after overnight fasting before loading and 4 hours after loading. Absorption of methionine was verified by determination of methionine[18] in the fasting and postload samples. The values for plasma methionine 4 hours after loading were higher than 164 μmol/L in all subjects.

Biochemical Analysis

Blood was routinely collected into three evacuated tubes containing either monobromobimane (mBrB) or *N*-ethylmaleimide (NEM) as thiol-derivatizing reagent or no additions. The blood was immediately centrifuged at 10 000*g* for 1 minute at room temperature to remove blood cells.

From the analysis of blood collected in a solution containing mBrB we obtained reduced thiols, analysis of blood collected into NEM gave the oxidized forms, and total amount of thiol components was assayed in nontreated plasma. The protein-bound form is calculated by subtracting reduced and free oxidized forms from the total amount.

Details on the construction and performance of these assays are described elsewhere.[13]

Plasma samples were frozen and stored at −70°C until analysis. Vitamin B_6 was measured as pyridoxal 5′-phosphate with an enzymic method.[19] Serum cobalamin and serum folate were measured by SimulTRAC-SNB Radioassay Kit from Becton Dickinson. Serum cholesterol and triglycerides were measured with an enzymic colorimetric assay (Boehringer-Mannheim automated analyses for Hitachi system 717, Diagnostica). HDL cholesterol was measured after lipoproteins containing apolipoprotein B were precipitated with phospho-

TABLE 2. Concentrations of Various Forms of Homocysteine and Other Aminothiols in Plasma From Patients and Control Subjects Before and After Methionine Loading

	Fasting		After Loading	
	Patients	Control Subjects	Patients	Control Subjects
Males				
Homocysteine				
Total	15.7 (13.6-18.1)	11.7 (10.8-12.7)	39.8 (35.5-44.7)	28.1 (26.1-30.3)
Reduced	0.07 (0.05-0.09)	0.04 (0.03-0.05)	0.45 (0.36-0.56)	0.27 (0.22-0.34)
Oxidized	1.81 (1.49-2.20)	1.25 (1.11-1.41)	7.12 (6.16-8.23)	4.31 (3.74-4.96)
Protein-bound	13.6 (11.7-15.8)	10.3 (9.40-11.3)	32.6 (29.0-36.7)	23.6 (21.9-25.4)
R/T × 100*	0.5 (0.4-0.6)	0.4 (0.3-0.5)	1.1 (0.9-1.3)	1.0 (0.8-1.2)
Cysteine				
Total	321.9 (301.2-342.6)	292.9 (280.0-305.8)	275.7 (261.7-289.7)	243.8 (232.8-254.8)
Reduced	11.4 (10.0-12.8)	14.5 (13.0-16.1)	15.6 (13.6-17.6)	18.2 (15.9-20.5)
Oxidized	94.4 (85.6-103.0)	88.2 (83.0-93.4)	95.0 (87.0-103.1)	84.9 (78.8-91.0)
Protein-bound	216.2 (198.2-234.3)	190.2 (176.7-203.7)	165.1 (154.9-175.3)	140.7 (128.9-152.5)
R/T × 100*	3.6 (3.1-4.1)	5.0 (4.4-5.6)	5.7 (5.0-6.4)	7.7 (6.5-8.9)
Cysteinylglycine				
Total	26.7 (24.7-28.7)	25.8 (23.7-27.9)	23.3 (21.3-25.3)	22.6 (20.6-24.6)
Reduced	3.70 (3.25-4.15)	3.86 (3.41-4.31)	4.13 (3.61-4.65)	3.93 (3.45-4.41)
Oxidized	5.12 (4.42-5.82)	5.42 (4.99-5.85)	4.91 (4.29-5.53)	4.61 (4.19-5.03)
Protein-bound	17.7 (16.1-19.3)	16.7 (15.1-18.3)	13.9 (12.4-15.4)	13.9 (12.3-15.5)
R/T × 100*	14.0 (12.6-15.4)	15.1 (13.4-16.8)	18.0 (16.0-20.0)	18.0 (15.5-20.5)
Females				
Homocysteine				
Total	14.6 (12.2-17.5)	11.2 (10.1-12.5)	36.0 (31.6-41.0)	33.1 (29.5-37.2)
Reduced	0.07 (0.05-0.09)	0.06 (0.05-0.07)	0.51 (0.40-0.64)	0.50 (0.38-0.65)
Oxidized	1.80 (1.54-2.10)	1.31 (1.16-1.48)	6.12 (5.31-7.05)	4.70 (3.98-5.55)
Protein-bound	12.7 (10.6-15.3)	9.83 (8.82-10.98)	28.6 (25.2-32.5)	27.1 (24.3-30.2)
R/T × 100*	0.5 (0.4-0.6)	0.5 (0.4-0.6)	1.4 (1.2-1.6)	1.5 (1.2-1.9)
Cysteine				
Total	311.3 (292.7-329.9)	303.3 (289.4-317.2)	274.9 (258.9-290.9)	266.4 (254.6-278.2)
Reduced	11.3 (9.7-12.9)	12.9 (11.4-14.4)	18.4 (15.9-20.9)	21.09 (19.08-23.10)
Oxidized	90.9 (84.7-97.1)	88.0 (81.8-94.2)	96.7 (90.9-102.5)	89.0 (82.0-96.0)
Protein-bound	209.1 (193.1-225.1)	202.4 (190.3-214.5)	162.0 (149.1-174.9)	156.4 (145.7-167.1)
R/T × 100*	3.7 (3.2-4.2)	4.4 (3.8-5.0)	6.7 (5.8-7.6)	8.0 (7.2-8.8)
Cysteinylglycine				
Total	25.7 (23.5-27.9)	28.4 (26.5-30.3)	20.0 (18.2-21.8)	22.8 (21.2-24.4)
Reduced	3.33 (2.88-3.78)	3.94 (3.55-4.33)	3.47 (2.96-3.98)	4.39 (3.95-4.83)
Oxidized	5.42 (4.82-6.02)	5.61 (4.97-6.25)	5.22 (4.67-5.77)	5.15 (4.49-5.81)
Protein-bound	17.2 (15.6-18.8)	18.7 (17.1-20.3)	11.9 (10.6-13.2)	13.5 (12.1-14.9)
R/T × 100*	13.0 (11.5-14.5)	14.3 (12.7-15.9)	17.4 (15.0-19.8)	20.0 (17.6-22.4)

The concentration unit is μmol/L. Data are given as mean values with 95% confidence intervals for the means in parentheses. n=35 for male patients, 30 for female patients, 34 for male control subjects, and 31 for female control subjects.
*R/T × 100 amount of reduced divided by total (reduced/total ratio) multiplied by 100.

tungstate/magnesium chloride. LDL cholesterol was calculated according to Friedewald's formula.[20]

Statistical Analysis

The SYSTAT statistical program, version 5.2, for Macintosh computer (Systat, Inc) was used for data analysis. All parameters were tested for goodness of fit to normal distribution as raw data or after logarithmic transformation with a Q-Q plot.[21] Total, reduced, oxidized, and protein-bound Hcy, the reduced/total Hcy ratio, triglycerides, cobalamin, and folate were found to be best fit to a log-normal distribution. The other parameters were analyzed as nontransformed data. The influence of disease, gender, and smoking on aminothiol forms was tested for in a multivariate model (MANOVA), which estimated possible interactions between the variables.

Protein-bound Cys before and after methionine loading was evaluated by paired t test and serum lipids in patients and control subjects were compared by two-sample t test.

We determined the correlation between vitamins and various forms of Hcy using univariate and stepwise multivariate regression analysis.

Results

Population Characteristics

At follow-up, triglycerides were higher and HDL cholesterol was lower in both male and female patients compared with the respective control groups ($P<.05$). Total cholesterol and LDL cholesterol were significantly higher only in female patients compared with female control subjects ($P<.02$).

Among the patients, 63 had a history of smoking and 35 were current smokers, whereas the corresponding numbers for control subjects were 37 and 19. Seven of the patients had diabetes compared with only 1 female in the control group. Fifteen patients and 8 control subjects had hypertension (Table 1).

Aminothiols in Patients Versus Control Subjects

In fasting subjects, all Hcy forms (total Hcy, reduced, oxidized, and protein-bound forms) were significantly higher in patients compared with control subjects. Similarly, total and protein-bound Cys were significantly

TABLE 3. Differences in Various Forms of Homocysteine and Cysteine in Plasma in Relation to Sex, Vascular Disease, and Smoking

	Homocysteine					Cysteine				
Test	Total *P*	Reduced *P*	Oxidized *P*	Protein Bound *P*	R/T* Ratio *P*	Total *P*	Reduced *P*	Oxidized *P*	Protein Bound *P*	R/T Ratio *P*
Fasting										
Patients vs control subjects	<.001	.01	<.001	.01	NS†	.04	.001	NS	.04	<.001
Men vs women	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Smokers vs nonsmokers	.01	NS	.006	.02	NS	NS	NS	NS	NS	NS
Interaction disease and sex	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Postmethionine load										
Patients vs control subjects	.002	NS	<.001	.007	NS	.002	.01	.03	.003	<.001
Men vs women	NS	.01	NS	NS	.02	.06	.03	NS	NS	NS
Smokers vs nonsmokers	.02	.03	.01	.03	NS	NS	NS	NS	NS	NS
Interaction disease and sex	.005†	.02†	.05†	.004‡	NS	NS	NS	NS	NS	NS

*R/T indicates reduced/total.
† Not significant ($P>.05$).
‡The difference in the concentrations of homocysteine species between patients and control subjects was smaller in women than in men.

higher in the patient group, whereas reduced Cys was higher in control subjects compared with the patients. There were no sex differences (Tables 2 and 3).

After methionine loading, all Hcy forms increased threefold to sevenfold. The largest increase was observed for the reduced form (Table 2). The differences in aminothiol levels between patients and control subjects resembled that observed after fasting. All forms of Hcy and Cys, except reduced Cys, were higher in patients than in control subjects. Notably, reduced Cys after loading was higher in control subjects than in patients, in both sexes (Tables 2 and 3).

The postload values showed some sex differences. The difference in total and protein-bound Hcy between patients and control subjects was less in women (6% to 9%) than in men (40%), and there was no significant difference in reduced Hcy between female patients and control subjects. Furthermore, reduced Cys was significantly higher in women than in men (Tables 2 and 3).

Total CysGly (Table 2) was not significantly different ($P>.05$) between patients and control subjects.

The most notable findings described above (Tables 2 and 3) are illustrated in the cumulative frequency distribution curves presented in Fig 1. The whole frequency distribution curves for total Hcy and total Cys during fasting and after loading in men, and the curves for total Hcy for women, are displaced to the right in patients relative to control subjects. The lower panels of Fig 1 demonstrate the low levels of reduced Cys in both male and female patients, during fasting and after methionine loading, compared with control subjects.

We found a significant positive correlation between total Hcy during fasting and after methionine loading both in patients ($R=.68$, $P<.001$) and control subjects ($R=.56$, $P<.001$), and the postload values did not discriminate better between the two groups than the fasting levels did (Table 2, Fig 1). However, fasting and postload values did not give overlapping results. When the upper limits of the 95% confidence interval of the individual observations of control subjects were taken as cutoff points, 8 of 35 male patients had a normal fasting level and elevated postload total Hcy, and 4 of 35 had an elevated fasting level and normal postload level. The corresponding fractions for female patients were 0/30 and 3/30, respectively.

Smoking

We have previously investigated total Hcy in 58 subjects of this patient population and found that total fasting Hcy was higher in smokers than in nonsmokers.[22] Data presented here show that this could be ascribed to elevation of both the protein-bound and oxidized forms. In addition, we could also demonstrate that the postload levels of all Hcy forms were significantly higher in smokers than in nonsmokers. Smoking did not influence plasma Cys forms (Table 3). Notably, while the number of smokers was somewhat higher among patients than control subjects (Table 1), smoking and disease independently influenced plasma aminothiols (Table 3).

Protein Binding of Aminothiols

There was a positive correlation between protein-bound Hcy and protein-bound Cys in fasting male ($r=.58$, $P=.001$) and female ($r=.47$, $P=.005$) control subjects whereas only a trend toward a positive relation was found in fasting male ($r=.30$, $P=.09$) and female ($r=.18$, $P=.35$) patients (Fig 2).

In female as well as male patients and control subjects, protein-bound Hcy increased and protein-bound Cys decreased ($P<.001$) after methionine loading (Table 2). In almost every individual within all four subgroups, methionine loading caused a marked drop in protein-bound Cys (Fig. 2).

Reduced Aminothiols and Redox Status

In both patients and control subjects, reduced Hcy was low during fasting and increased markedly after methionine loading (Table 2). After methionine loading, reduced Hcy was positively correlated to total Hcy in male ($r=.36$, $P<.05$) and female ($r=.38$, $P<.05$) control subjects and male ($r=.54$, $P<.005$) and female ($r=.77$, $P<.001$) patients (data not shown).

We investigated the relation between the reduced/total ratio (the fraction of the total amount that exists in the reduced form) for Hcy, Cys, and CysGly in patients

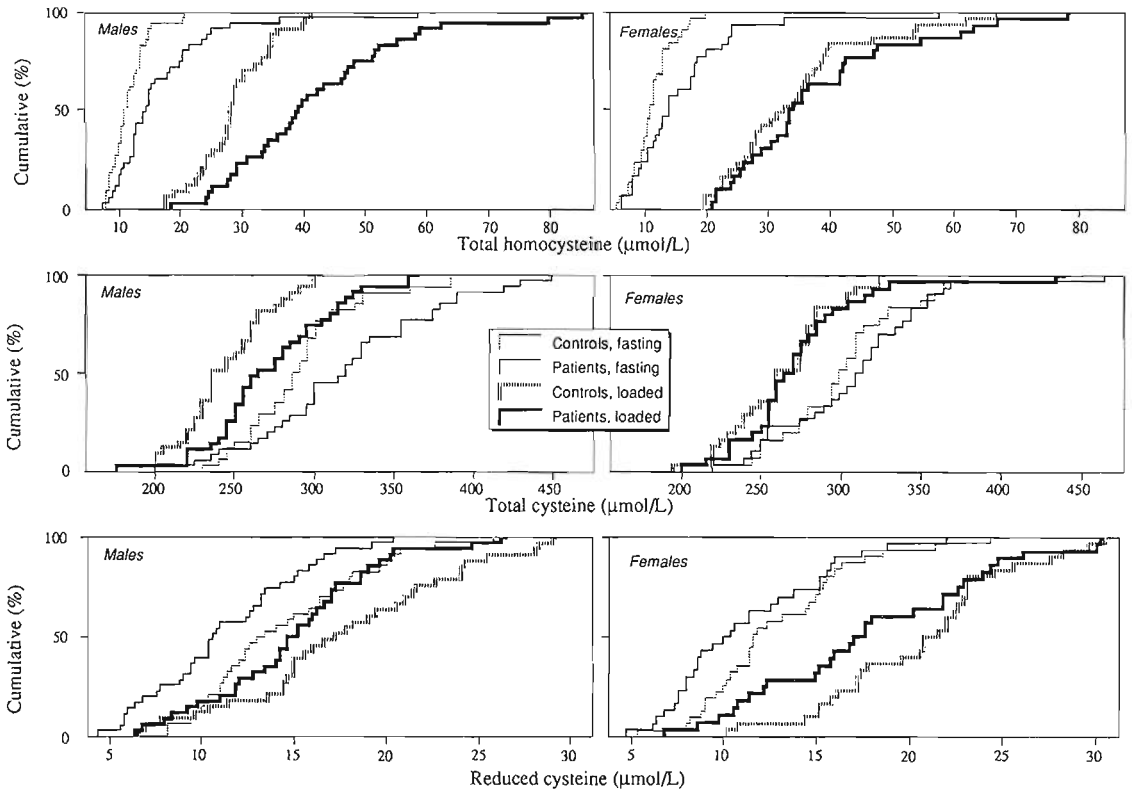

FIG 1. Graphs show cumulative frequency distribution of total homocysteine, total cysteine, and reduced cysteine in male and female patients and control subjects.

and control subjects after methionine loading. In both groups, we found a significant linear relation between the reduced/total ratio for Hcy and Cys; the relation between the reduced/total ratio for Hcy and CysGly was weaker. The ratios for Cys and CysGly also correlated. These relations for patients are shown in Fig 3. Essentially the same results were obtained for the control subjects (data not shown).

Correlations With Vitamins

None of the patients or control subjects had serum cobalamin below normal (<120 nmol/L), and 10 patients and no control subjects had serum folate below normal (<5 nmol/L).

A dietary assessment was done with respect to vitamin intake at follow-up. Eight of 65 patients and 9 of 61 control subjects had vitamin supplementation. However,

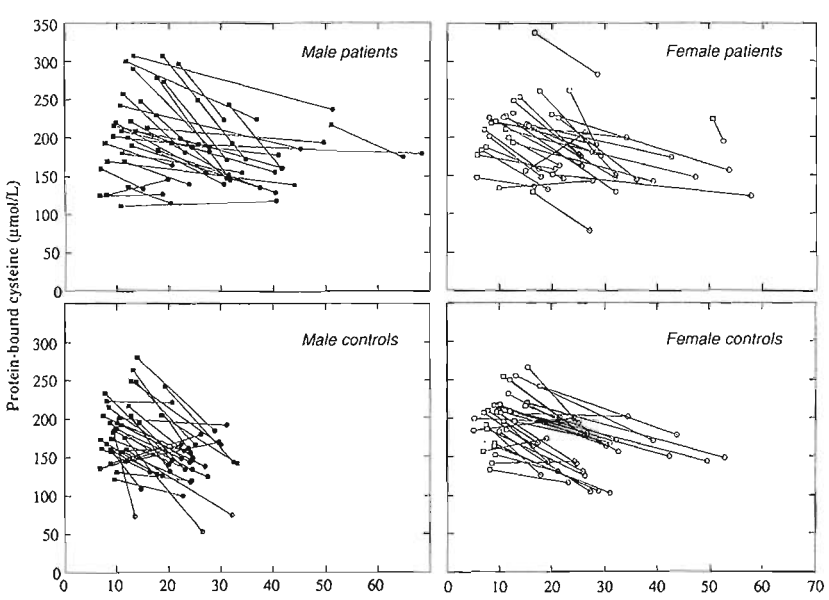

FIG 2. Plots show relation between protein-bound homocysteine (Hcy) and protein-bound cysteine (Cys) in the fasting state (■, □) and after methionine loading (●, ○). The lines connect the data points for each individual and indicate the increase in protein-bound Hcy and decline in protein-bound Cys after methionine loading.

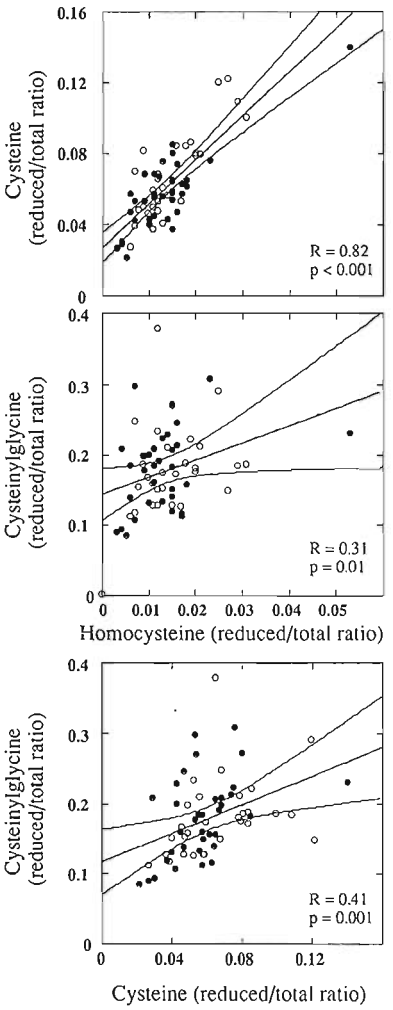

FIG 3. Plots show relation between redox status of homocysteine, cysteine, and cysteinylglycine in plasma from patients after methionine loading. The fraction existing in the reduced form is calculated as the amount of reduced form divided by the total amount, ie, the reduced/total ratio. ● indicates men; ○, women.

the levels of total fasting Hcy and total fasting Cys were not different in the subjects taking and not taking vitamins (data not shown).

We tested for the relation between various forms of Hcy and serum folate, serum cobalamin, and vitamin B_6 using regression analysis. Serum folate was negatively correlated with total and oxidized Hcy in all four groups except in female control subjects and was negatively correlated with protein-bound Hcy in male patients. Serum cobalamin and vitamin B_6 showed a negative correlation only with oxidized Hcy, and this relation was confined to male patients (cobalamin) and to male patients and female control subjects (vitamin B_6) (Table 4).

Discussion

Study Design

This is a case-control study including most patients operated on for early-onset peripheral vascular disease in two hospitals in Stockholm over a 12-year period.

Determination of redox status of three aminothiols in plasma is a cumbersome procedure requiring immediate sample processing[13]; stored plasma cannot be used. This strictly limits the number of both patients and control subjects included in this study. Because of limited recruitment of patients, we had to collect patients with a history of early-onset vascular disease, and follow-up and blood sampling were 0 to 11 years (mean, 6 years) after surgery. This time interval may introduce an error, because plasma Hcy changes with age[23] and possibly because of altered lifestyle, in particular, vitamin intake. However, the change in plasma Hcy over a 10-year period is limited (about 10%) and not significant.[23] Furthermore, in both healthy subjects and patients with cardiovascular disease, there is a highly significant correlation between plasma Hcy in fresh samples and old plasma samples from the same individuals stored for 6 to 16 years.[24] Only 8 patients were regularly taking vitamin supplements and the plasma Hcy was not significantly lower in this subgroup.

The patients were compared with age- and sex-matched control subjects selected from the population

TABLE 4. Regression Analysis for the Relation Between Various Homocysteine Species and Vitamins in Plasma From Fasting Patients and Control Subjects*

Variables			Female Control Subjects (n=31)	Female Patients (n=30)	Male Control Subjects (n=34)	Male Patients (n=35)
Dependent	Independent	Analysis	*P*			
Total	Folate	Univariate	.13	<.0025	<.05	<.001
		Multivariate	NS†	<.0025	<.05	<.001
	Vitamin B_6	Univariate	<.05	NS	NS	<.025
		Multivariate	<.05	NS	NS	NS
Oxidized	Folate	Univariate	<.005	<.0025	<.001	<.01
		Multivariate	NS	<.0025	<.05	<.001
	Cobalamin	Univariate	<.025	NS	NS	<.025
		Multivariate	NS	NS	NS	<.025
	Vitamin B_6	Univariate	<.005	NS	NS	<.05
		Multivariate	<.005	NS	NS	<.05
Protein-bound	Folate	Univariate	NS	NS	.05	<.001
		Multivariate	NS	NS	.05	<.001
	Vitamin B_6	Univariate	NS	NS	NS	<.025
		Multivariate	NS	NS	NS	NS
Reduced/total ratio	Vitamin B_6	Univariate	NS	NS	NS	<.025

*The effect of folate, cobalamin, and vitamin B_6 on various homocysteine species (total, reduced, oxidized, protein-bound, and reduced/total ratio) was evaluated using univariate analysis, and in case two or more independent variables were effective, these were included in a multivariate analysis.

†NS indicates not significant.

register. Since age and sex in addition to vascular disease are known to correlate with the plasma Hcy levels,[5] these parameters were included in the statistical model (Table 3). We also tested for smoking and its interactions (Table 3), because smoking is a particularly strong risk factor for peripheral vascular disease[25] and has been found to influence plasma Hcy in two recent studies.[26,27] The small size of the sample matrix did not justify the inclusion of additional dimensions such as serum lipids, diabetes, and hypertension, which in most studies have been found not to influence plasma Hcy level.[5]

Normal Hcy Values and Definition of Hyperhomocysteinemia

The mean values for total Hcy in healthy men (11.7 μmol/L) and women (11.2 μmol/L) reported in the present work (Table 2) equal the mean total Hcy (11.58 μmol/L) reported for a large population (n=3000) of healthy men aged 40 to 42 years[8] and normal values published by others.[23]

There is some variability of total Hcy relative to age and sex, and the normal range for total Hcy has been somewhat arbitrarily set at 5 to 15 μmol/L.[8] Kang et al[6] suggested the term hyperhomocysteinemia for total Hcy above normal and defined moderate hyperhomocysteinemia as levels up to 30 μmol/L. These are the levels often encountered in subjects without known defects in Hcy metabolism and in patients with premature cardiovascular disease.[5] Intermediate hyperhomocysteinemia, defined as levels between 30 and 100 μmol/L, is often present in patients with cobalamin or folate deficiencies, whereas severe hyperhomocysteinemia, defined as >100 μmol/L, is usually confined to patients with inborn errors of Hcy metabolism, ie, homocystinuria.[8]

Aminothiol Forms in Patients and Control Subjects

The present investigation demonstrates that fasting total Hcy is 34% to 42% higher in female and male patients with early-onset peripheral vascular disease than in matched control subjects, a difference that is highly significant. Similar differences were found after a methionine loading test (Tables 2 and 3, Fig 1). These data are consistent with the results from several epidemiological studies, showing that the amount of total plasma Hcy in a population with cardiovascular disease is about 30% higher than in healthy subjects.[5,6]

In some early works[28-31] and one recent study[32] the Cys-Hcy mixed disulfide, which corresponds to free oxidized Hcy in the present work, was measured and found to be elevated in plasma from patients with cardiovascular disease. However, the different Hcy forms in plasma from patients with hyperhomocysteinemia and cardiovascular disease have not been investigated previously.

Table 2 shows that all Hcy forms were increased in fasting patients compared with control subjects. Particularly in male patients, reduced Hcy was markedly elevated. A similar difference between patients and control subjects was observed after methionine loading, except that the amount of reduced Hcy in female patients equaled that found in female control subjects.

We also measured different forms of plasma Cys and CysGly and found that total, protein-bound, and oxidized Cys were significantly higher in both female and male patients compared with control subjects, both during fasting and after methionine loading (Tables 2 and 3, Fig 1). This finding agrees with the results of a Japanese study[33] showing that total and free Cys (and Hcy) in plasma were elevated in 45 patients with cerebral infarction.

Reduced Cys (and accordingly the reduced/total ratio) was the only aminothiol component in plasma that was significantly higher in control subjects than in patients. This was a consistent finding in men as well as women and was found in fasting as well as after methionine loading (Tables 2 and 3, Fig 1). The low level of reduced Cys and the low reduction state of Cys in patients with peripheral vascular disease may reflect impaired redox thiol status in at least some of these patients. Conceivably, other thiols may be involved, as recently demonstrated by low sulfhydryl reactivity of albumin in patients with coronary artery disease.[34]

Redox Status and Protein Binding of Aminothiols

We have previously studied the dynamic relation existing between the reduced, oxidized, and protein-bound forms of various aminothiols in human plasma. These studies included healthy subjects with a transiently increased plasma Hcy due to a methionine[14] or Hcy[15] load, 8 homocystinuric patients,[16] and 13 patients with hyperhomocysteinemia due to cobalamin deficiency.[17] These clinical data demonstrated that reduced Hcy is low under physiological conditions but increases as a function of total Hcy, especially at high (>100 μmol/L) levels; that alterations in the redox status of Hcy affected the redox status of other aminothiol components in plasma; and finally, that high levels of Hcy displace Cys from the binding site in plasma.[14-17]

One objective of the present work was to investigate whether these relations between plasma aminothiols observed under conditions of intermediate and severe hyperhomocysteinemia[14-17] also exist in patients with vascular disease having moderate hyperhomocysteinemia (15 to 30 μmol/L) during fasting and in healthy subjects with normal Hcy level. Knowledge about secondary effects on other aminothiol components in plasma may point to future directions of research on processes responsible for the vascular lesions in patients with hyperhomocysteinemia.

First, we showed that reduced Hcy increased as a function of total Hcy in both patients and control subjects (data not shown). Second, we could demonstrate a positive correlation between the reduced/total ratio for Hcy and Cys, between the ratio for Hcy and CysGly, and between the ratio for Cys and CysGly in both control subjects (data not shown) and patients (Fig 3). For a particular aminothiol component, this ratio represents the fraction of the total amount (sum of reduced, oxidized, and protein-bound) existing in the reduced form and is a measure of its redox status in plasma. The positive correlation between the ratios suggests interaction between these aminothiol forms through redox reaction and thiol-disulfide exchange. Thus, altered redox status of plasma Hcy is not an isolated event but affects the redox status of related aminothiol components.

The positive correlation between protein-bound Hcy and protein-bound Cys in the fasting state may reflect that both Hcy and Cys are products of the transsulfuration pathway.[7] The marked increase in bound Hcy after methionine loading caused a drop in bound Cys in most patients and control subjects (Fig 2). Similarly, in pa-

tients with intermediate and severe hyperhomocysteinemia, a negative correlation between protein-bound Hcy and Cys has been found.[16,17,35] These relations between protein binding of Hcy and Cys may be explained by displacement of bound Cys by Hcy. This explanation is supported by the presence in plasma of saturable[35] binding sites that preferentially interact with Hcy.[35-37]

Sex

It has been suggested that efficient methionine metabolism in premenopausal women offers protection against cardiovascular diseases.[38] This hypothesis was based on the findings of lower levels of fasting and postload Hcy-Cys mixed disulfide (corresponding to oxidized Hcy in the present work) in premenopausal women than in postmenopausal women and men.

Marked sex-related differences in Hcy levels[38] have recently been contested by Andersson et al.[23] Notably, they also observed that about 30% of healthy postmenopausal women responded to methionine loading with distinctly higher values than men and premenopausal women. We made similar observations in the present study. Total fasting Hcy in men was only marginally higher than in women (Table 2). Furthermore, female control subjects had higher postload total Hcy than men (Table 1), and 5 (age, 46 to 60 years) out of 31 female control subjects and no male control subjects had postload protein-bound Hcy higher than 34 μmol/L (Fig 2).

The differences between patients and control subjects in the levels of all Hcy forms after loading were larger in men than in women (Tables 2 and 3), as revealed by a statistical interaction between disease and sex (Table 3). This is also illustrated for total Hcy in the frequency distribution graph (Fig 1). Furthermore, both reduced Hcy and reduced Cys after loading were significantly higher in women than in men (Tables 2 and 3).

Vitamins

Total, protein-bound, and oxidized Hcy were negatively correlated with serum folate in most groups, whereas serum cobalamin was negatively correlated only with oxidized Hcy in male patients and vitamin B_6 with oxidized Hcy in male patients and female control subjects (Table 4). These data agree with the observations that serum folate is a strong predictor of plasma Hcy, whereas weaker correlations with serum cobalamin and vitamin B_6 are occasionally found.[39,40] However, the design of the present study was not optimized to study the relation between Hcy forms and vitamin status, especially because only a few vitamin-deficient subjects were included.

Possible Mechanisms

Several mechanisms, involving diverse targets like lipoprotein(a),[41] endothelium anticoagulant mechanisms,[42,43] endothelial cells, coagulation factors, platelets, and LDL, have been suggested for the vascular damage caused by elevated Hcy.[5] In vitro oxidative modification of LDL,[44,45] inhibition of endothelium anticoagulant mechanisms,[43] and endothelial cell injury[46,47] have been demonstrated in the presence of reduced Hcy and are believed to be mediated by oxygen-derived free radicals formed during oxidation of Hcy catalyzed by a redox metal.

Various forms of Hcy should be considered as mediators of the possible atherogenic effect of hyperhomocysteinemia. The fraction associated with plasma protein(s) is probably biologically inactive, and the free forms (reduced and oxidized) are the most likely candidates.

Aminothiols, like Hcy and Cys, may function as pro-oxidants at low concentration and antioxidants at high concentration,[45] but their redox properties may also be influenced by the pH[45] and the composition[48] of the medium in which the reactions take place. In biochemical model reactions, Hcy, but not Cys, has pro-oxidant potentials, and physiological levels of Cys even antagonize the oxidative damage by Hcy.[49] Thus, one may speculate whether elevated levels of reduced Hcy in patients with early-onset peripheral vascular disease (Table 2) have pro-oxidant effect–causing vascular lesions, whereas the low level of reduced Cys in these patients (Table 2, Fig 1) may reflect impaired mechanisms responsible for scavenging reactive oxygen species.

Reduced Cys is the most abundant low-molecular-weight thiol in plasma,[13] and the reduced/total ratio may reflect the overall antioxidant status of plasma.[50]

Summary and Conclusions

In patients with early-onset peripheral arteriosclerosis, all Hcy and Cys forms in plasma are elevated above normal, except reduced Cys, which is consistently lower than in control subjects. An attractive hypothesis is that reduced Hcy has pro-oxidant properties provoking vascular lesions, whereas reduced Cys may serve as a protective factor.

Both in patients and in healthy control subjects, the redox status and the protein binding of different aminothiols in plasma were related in a manner (Figs 2 and 3) suggesting that the aminothiols undergo redox cycling and thiol-disulfide exchange reactions. These processes probably also involve other thiol components and may also interact with membrane-associated antioxidant systems.[51,52] Thus, altered redox status or concentrations of aminothiols in plasma may directly or indirectly cause the damage responsible for arteriosclerosis. Whatever the mechanism, the present work demonstrates that alteration in total Hcy in patients with vascular disease may represent a single component in a complex interactive system.

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References

1. Mudd SH, Levy HL, Skovby F. Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic Basis of Inherited Disease*. New York, NY: McGraw-Hill; 1989:693-734.
2. Stampfer MJ, Malinow MR, Willett WC, Newcomer LM, Upson B, Ullmann D, Tishler PV, Hennekens CH. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. *JAMA*. 1992;268:877-881.
3. Arnesen E, Refsum H, Bønaa KH, Ueland PM, Førde OH, Nordrehaug JE. The Tromsø study: a population based prospective study of serum total homocysteine and coronary heart disease. *Int J Epidemiol*. In press.
4. Malinow MR. Hyperhomocyst(e)inemia: a common and easily reversible risk factor for occlusive atherosclerosis. *Circulation*. 1990; 81:2004-2006. Editorial.
5. Ueland PM, Refsum H, Brattström L. Plasma homocysteine and cardiovascular disease. In: Francis RBF, ed. *Atherosclerotic Cardiovascular Disease, Hemostasis, and Endothelial Function*. New York, NY: Marcel Dekker Inc; 1992:183-236.

6. Kang S-S, Wong PWK, Malinow MR. Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. *Annu Rev Nutr.* 1992; 12:279-298.
7. Finkelstein JD. Methionine metabolism in mammals. *J Nutr Biochem.* 1990;1:228-237.
8. Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A, Allen RH. Total homocysteine in plasma or serum: methods and clinical applications. *Clin Chem.* 1993;39:1764-1779.
9. Refsum H, Helland S, Ueland PM. Radioenzymic determination of homocysteine in plasma and urine. *Clin Chem.* 1985;31:624-628.
10. Gupta VJ, Wilcken DEL. The detection of cysteine-homocysteine mixed disulphide in plasma of normal fasting man. *Eur J Clin Invest.* 1978;8:205-207.
11. Ueland PM, Refsum H. Plasma homocysteine, a risk factor for vascular disease: plasma levels in health, disease, and drug therapy. *J Lab Clin Med.* 1989;114:473-501.
12. Savage DG, Lindenbaum J, Stabler SP, Allen RH. Sensitivity of serum methylmalonic acid and total homocysteine determinations for diagnosing cobalamin and folate deficiencies. *Am J Med.* 1994; 96:239-246.
13. Mansoor MA, Svardal AM, Ueland PM. Determination of the in vivo redox status of cysteine, cysteinylglycine, homocysteine and glutathione in human plasma. *Anal Biochem.* 1992;200:218-229.
14. Mansoor MA, Svardal AM, Schneede J, Ueland PM. Dynamic relation between reduced, oxidized and protein-bound homocysteine and other thiol components in plasma during methionine loading in healthy men. *Clin Chem.* 1992;38:1316-1321.
15. Mansoor MA, Guttormsen AB, Fiskerstrand T, Refsum H, Ueland PM, Svardal AM. Redox status and protein-binding of plasma aminothiols during the transient hyperhomocysteinemia following homocysteine administration. *Clin Chem.* 1993;39:980-985.
16. Mansoor MA, Ueland PM, Aarsland A, Svardal AM. Redox status and protein-binding of plasma homocysteine and other aminothiols in patients with homocystinuria. *Metabolism.* 1993;42:1481-1485.
17. Mansoor MA, Ueland PM, Svardal AM. Redox status and protein-binding of plasma homocysteine and other aminothiols in patients with hyperhomocysteinemia due to cobalamin deficiency. *Am J Clin Nutr.* 1994;59:631-635.
18. Krishnamurti CR, Heindze AM, Galzy G. Application of reversed-phase high-performance liquid chromatography using pre-column derivatization with o-phthaldialdehyde for the quantitative analysis of amino acids in adult and fetal sheep plasma, animal feeds and tissues. *J Chromatogr.* 1984;315:321-331.
19. Hamfelt A. A simplified method for determination of pyridoxal phosphate in biological samples. *Ups J Med Sci.* 1986;91:105-109.
20. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem.* 1972;18:499-502.
21. Johnson RA, Wichern DW. The multivariate normal distribution. In: Johnson RA, Wichern DW, eds. *Applied Multivariate Statistical Analysis.* Englewood Cliffs, NJ: Prentice-Hall Inc; 1982:150-173.
22. Bergmark C, Mansoor MA, Swedenborg J, de Faire U, Svardal AM, Ueland PM. Hyperhomocysteinemia in patients operated for lower extremity ischaemia below the age of 50: effect of smoking and extent of disease. *Eur J Vasc Surg.* 1993;7:391-396.
23. Andersson A, Brattström L, Israelsson B, Isaksson A, Hamfelt A, Hultberg B. Plasma homocysteine before and after methionine loading with regard to age, gender, and menopausal status. *Eur J Clin Invest.* 1992;22:79-87.
24. Israelsson B, Brattström L, Refsum H. Homocysteine in frozen plasma samples—a short cut to establish hyperhomocysteinaemia as a risk factor for arteriosclerosis. *Scand J Clin Lab Invest.* 1993; 53:465-469.
25. Fowkes FGR. Aetiology of peripheral atherosclerosis: smoking seems especially important. *Br Med J.* 1989;298:405-406.
26. Williams RR, Malinow MR, Hunt SC, Upson B, Wu LL, Hopkins PN, Stults BN, Kuida H. Hyperhomocyst(e)inemia in Utah siblings with early coronary disease. *Coron Artery Dis.* 1990;1:681-685.
27. Wu LL, Wu J, Hunt SC, James BC, Vincent GM, Williams RR, Hopkins PN. Plasma homocyst(e)ine as a risk factor for early familial coronary artery disease. *Clin Chem.* 1994;40:552-561.
28. Wilcken DEL, Wilcken B. The pathogenesis of coronary artery disease: a possible role for methionine metabolism. *J Clin Invest.* 1976;57:1079-1082.
29. Braatström LE, Hardebo JE, Hultberg BL. Moderate homocysteinemia—a possible risk factor for arteriosclerotic cerebrovascular disease. *Stroke.* 1984;15:1012-1016.
30. Boers GHJ, Smals AGH, Trijbels FJM, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. *N Engl J Med.* 1985;313:709-715.
31. Murphy-Chutorian DR, Wexman MP, Grieco AJ, Heininger JA, Glassman E, Gaull GE, Ng SKC, Feit F, Wexman K, Fox AC. Methionine intolerance: a possible risk factor for coronary artery disease. *J Am Coll Cardiol.* 1985;6:725-730.
32. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia: an independent risk factor for vascular disease. *N Engl J Med.* 1991;324:1149-1155.
33. Araki A, Sako Y, Fukushima Y, Matsumoto M, Asada T, Kita T. Plasma sulfhydryl-containing amino acids in patients with cerebral infarction and in hypertensive subjects. *Atherosclerosis.* 1989;79: 139-146.
34. Kadota K, Yui Y, Hattori R, Murohara Y, Kawai C. Decreased sulfhydryl groups of serum albumin in coronary artery disease. *Jpn Circ J.* 1991;55:937-941.
35. Wiley VC, Dudman NPB, Wilcken DEL. Interrelations between plasma free and protein-bound homocysteine and cysteine in homocystinuria. *Metabolism.* 1988;37:191-195.
36. Smolin LA, Benevenga NJ. Accumulation of homocyst(e)ine in vitamin B-6 deficiency: a model for the study of cystathionine β-synthase deficiency. *J Nutr.* 1982;112:1264-1272.
37. Smolin LA, Benevenga NJ. The use of cyst(e)ine in the removal of protein-bound homocysteine. *Am J Clin Nutr.* 1984;39:730-737.
38. Boers GH, Smals AG, Trijbels FJ, Leermakers AI, Kloppenborg PW. Unique efficiency of methionine metabolism in premenopausal women may protect against vascular disease in the reproductive years. *J Clin Invest.* 1983;72:1971-1976.
39. Ubbink JB, Vermaak WJH, van der Merwe A, Becker PJ. Vitamin B-12, vitamin B-6, and folate nutritional status in men with hyperhomocysteinemia. *Am J Clin Nutr.* 1993;57:47-53.
40. Selhub J, Jacques PF, Wilson PWF, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *JAMA.* 1993;270:2693-2698.
41. Harpel PC, Chang VT, Borth W. Homocysteine and other sulfhydryl compounds enhance the binding of lipoprotein(a) to fibrin: a potential biochemical link between thrombosis, atherogenesis, and sulfhydryl compound metabolism. *Proc Natl Acad Sci U S A.* 1992;89:10193-10197.
42. Hayashi T, Honda G, Suzuki K. An atherogenic stimulus homocysteine inhibits cofactor activity of thrombomodulin and enhances thrombomodulin expression in human umbilical vein endothelial cells. *Blood.* 1992;79:2930-2936.
43. Nishinaga M, Ozawa T, Shimada K. Homocysteine, a thrombogenic agent, suppresses anticoagulant heparan sulfate expression in cultured porcine aortic endothelial cells. *J Clin Invest.* 1993;92:1381-1386.
44. Heinecke JW, Rosen H, Suzuki LA, Chait A. The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. *J Biol Chem.* 1987;262:10098-10103.
45. Munday R. Toxicity of thiols and disulfides: involvement of free-radical species. *Free Radic Biol Med.* 1989;7:659-673.
46. Starkebaum G, Harlan JM. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. *J Clin Invest.* 1986;77:1370-1376.
47. Berman RS, Martin W. Arterial endothelial barrier dysfunction: actions of homocysteine and the hypoxanthine-xanthine oxidase free radical generating system. *Br J Pharmacol.* 1993;108:920-926.
48. Parthasarathy S. Oxidation of low-density lipoprotein by thiol compounds leads to its recognition by the acetyl LDL receptor. *Biochim Biophys Acta.* 1987;917:337-340.
49. Preibisch G, Küffner C, Elstner EF. Biochemical model reactions on the prooxidative activity of homocysteine. *Z Naturforsch [C].* 1993;48:58-62.
50. Halliwell B, Gutteridge JMC. The antioxidants of human extracellular fluids. *Arch Biochem Biophys.* 1990;280:1-8.
51. Di Mascio P, Murphy ME, Sies H. Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. *Am J Clin Nutr.* 1991; 53(suppl 1):194S-200S.
52. Liebler DC. The role of metabolism in the antioxidant function of vitamin E. *Crit Rev Toxicol.* 1993;23:147-169.